EXPERT REVIEWS

Anthrax, toxins and vaccines: a 125-year journey targeting *Bacillus anthracis*

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Bacillus anthracis is the causative agent of anthrax, a disease that plagues both humans and various animal species. Effective vaccines are available, but those approved for human use are crude culture supernatants that require multiple injections and a yearly boost. Many experts agree that it is now time for the next generation of human vaccines against anthrax. Accordingly, this review will succinctly focus upon: pathogenesis of *B. anthracis*, with particular emphasis upon the immune system; the pertinent biophysical nature of protective antigen, which includes how the protein toxin component affords protection as a vaccine target; alternative methods for improving protective antigen as an immunogen; and additional *B. anthracis* antigens that might further sustain protective titers in humans. In addition to a better understanding of the disease process elicited by *B. anthracis*, which will logically lead to better vaccines (and therapeutics), there also needs to be the same level of open-mindedness applied to the politics of anthrax.

KEYWORDS: anthrax • *Bacillus anthracis* • PA • pathogenesis • protective antigen • vaccine

The bacterial agent of anthrax was first described by a French physician, Casimir Davaine, in the blood of infected animals in 1850. However, it was nearly three decades later (1877) when this bacterium was definitively isolated and named *Bacillus anthracis* by Robert Koch, a German physician/microbiologist [1]. The link between a disease (anthrax) caused by one type of microorganism (*B. anthracis*) was conclusively established for the very first time. These basic tenets for pathogenic microbiology, known as Koch's postulates, are still applicable over 100 years after their inception. Since these early beginnings, additional medical history linked to *B. anthracis* coincides in many ways with vaccines that target anthrax (TABLE 1). This review will present the multiple attempts, by various international groups, to prevent anthrax in animals and humans by vaccination. Hopefully, the reader will be challenged to think of how existing anthrax vaccines, especially those meant for humans, can be improved with available knowledge/technology.

The many current anthrax vaccines are linked to Louis Pasteur's seminal experiments at Pouilly le-Fort in 1881, symbolizing the victory of science over a disease (anthrax) that ravaged European livestock at that time [2]. The vaccine schedule devised by Pasteur and colleagues was comprised of two injections administered at a 2-week interval. The inoculum consisted of attenuated *B. anthracis* that had been cultured at 42°C for 20 and 10 days for the first and second injections, respectively. Such growth conditions probably cured *B. anthracis* of the toxin-producing plasmid; however, very low numbers of fully virulent bacteria probably remained in the preparation. After this success, Pasteur's anthrax vaccine quickly gained fame and became widely used for livestock throughout Europe as well as South America. During the 1920s, this veterinary vaccine was further modified by the addition of glycerin to increase spore longevity. Saponin was added 10 years later to enhance immunogenicity, but unfortunately this detergent-like ingredient derived from plants also increased the risk of violent inflammatory responses.

It was during the 1930s that Max Sterne, a veterinarian of Austrian descent working at the Onderstepoort Veterinary Research Institute of South Africa, improved the anthrax vaccine by culturing a *B. anthracis* isolate for 24 h on 50% horse serum agar under a 30% CO_2 atmosphere [3]. The resultant spore-based inoculum

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14. ABSTRACT

Bacillus anthracis is the causative agent of anthrax, a disease that plagues both humans and various animal species. Effective vaccines are available, but those approved for human use are crude culture supernatants that require multiple injections and a yearly boost. many experts agree that it is now time for the next generation of human vaccines against anthrax. Accordingly, this review will succinctly focus upon: pathogenesis of B. anthracis, with particular emphasis upon the immune system; the pertinent biophysical nature of protective antigen, which includes how the protein toxin component affords protection as a vaccine target; alternative methods for improving protective antigen as an immunogen; and additional B. anthracis antigens that might further sustain protective titers in humans. In addition to a better understanding of the disease process elicited by B. anthracis, which will logically lead to better vaccines (and therapeutics), there also needs to be the same level of open-mindedness applied to the politics of anthrax.

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Table 1. Licensed anthrax vaccines.

Type	Name (country)	Antigens	Adjuvant	Route (doses)	Ref.
For human use					
Live vaccine	STI (Russia)	Spores (acapsulated strain)	None	id. (1)	[4]
PA-based supernatant	AVA (USA)	PA ± LF and EF	Aluminum hydroxide adsorbed	sc. (6)	[4]
	AVP (UK)	PA ± LF and EF	Alum precipitated	sc. (6)	[4]
	AVP + pertussis (UK)	PA ± LF and EF whole-cell pertussis vaccine	Alum precipitated Alum adsorbed	sc. (ND)	[171]
PA-based recombinant	rPA102*(USA)	rPA	Aluminum hydroxide adsorbed	sc. (3)	[126]
For veterinary use					
Live vaccine	Pasteur	Spores (atoxigenic/toxigenic strain mixture)	None	ND (2)	[4]
	Sterne	Spores (acapsulated strain)	None	sc. (1)	[4]

*Human use under clinical trial.
AVA: Anthrax vaccine adsorbed; AVP: Anthrax vaccine precipitated; EF: Edema factor; id.: Intradermal; LF: Lethal factor; ND.: Not determined; PA: Protective antigen; sc.: Subcutaneous.

has now become a stable, acapsulated strain (designated as 34F2) widely used to this day in the veterinary field. Perhaps it is via such long-term success enjoyed in the veterinary arena, with various herbivorous species, that modern researchers should take note for generating more efficacious anthrax vaccines for humans? Evidently a spore-based vaccine for anthrax has been used for humans in the former Soviet Union; however, this is considered unacceptable by many other countries due to cultural differences [4,5].

During the Cold War era that followed World War II, a further understanding of anthrax pathogenesis plus development of vaccines for humans was investigated vigorously by various countries. A push towards better anthrax vaccines was spurred on by the potential nefarious use of *B. anthracis* as a biowarfare agent against humans and not livestock. Animals had already been targeted, rather unsuccessfully for various reasons, during World War I [6]. An attempt to develop a vaccine for biodefense applications in the 1950s led to a rather crude, yet effective, acellular product consisting of *B. anthracis* culture supernatant proteins that include the aptly named protective antigen (PA). Open literature suggests that this type of research was simultaneously conducted by the UK and USA [7,8]. PA is a critical, cell-binding component for the transport of edema and lethal toxins into a targeted cell, as described in detail later. Worldwide interest in improved anthrax vaccines and therapeutics, of which many target PA, continues to this day.

Intriguingly, although a first-generation vaccine against anthrax is licensed in the USA and has been used on a large scale to immunize mill workers, researchers and service members, the efficacy, as well as safety, is still vigorously debated among various groups. The protective immune response of the currently licensed, culture supernatant-based vaccine is indeed hard to sustain, requiring numerous boosts [9]. Production of PA on a large scale can also be problematic [10] and, last but not least, vaccine efficacy against inhalational anthrax is not unequivocally established in humans [7]. However, studies in various animal models that include nonhuman primates do show very good efficacy against inhalational anthrax [11,12]. There are continued efforts today by many academic, government and industrial groups to generate new anthrax vaccines incorporating PA with or without other pertinent antigens of *B. anthracis* [13,14]. A major goal is to improve upon the existing, relatively crude *B. anthracis* vaccine for humans with longer-lasting, more-protective immunity that is efficacious not only against inhalational, but also cutaneous and intestinal, anthrax.

Various aspects of anthrax vaccines have already been reviewed by others [4,15–17], but our effort distinctly focuses upon the pathology and physiology of anthrax that may lead to new trials for future vaccine development. This current review will: present the main characteristics of anthrax; briefly delve into the pertinent biophysical nature of PA that includes how this toxin component affords protection as a vaccine target; and discuss alternative methods for improving PA as an immunogen. Additional discussion is dedicated to other promising *B. anthracis* antigens and immunization routes that may lead to longer-lasting, more-efficacious vaccines with available technology. Ultimately, we hope to shed further light and elicit further questioning upon the decades-old topic of targeting anthrax vaccines for humans.

What have we learned from pathogenesis models?

B. anthracis is a nonmotile, facultative anaerobe that occasionally infects humans; however, anthrax is most often a veterinary concern especially among field-grazed herbivores [18]. The ecological cycle of this Gram-positive bacterium involves proliferation of vegetative bacilli in the host and subsequent generation of dormant spores that remain viably persistent for decades in the soil. However, there is recent evidence that metabolically active (vegetative) forms of *B. anthracis* exist in soil [19]. Spores ingested by grazing herbivores typically constitute the infectious form of this pathogen.

B. anthracis has two main virulence factors thoroughly described throughout the literature: the poly-γ-D-glutamate acid (PGA) capsule (coded by the pXO2 plasmid) [20] and protein toxins

(coded by a temperature-sensitive pXO1 plasmid) [21]. The capsule plays an important role in establishing disease, as per protection of bacilli from complement fixation and phagocytes [22]. Toxins essentially provide the 'coup de grace' upon an infected host, leading to host death and sporulation of bacilli that perpetuates this deadly cycle. Association of enzymatically active moieties, lethal factor (LF) and edema factor (EF), with PA as a cell-surface binding component generates the lethal and edema toxins, respectively [23]. From a disease perspective, anthrax exhibits three clinical manifestations in humans [24]. These include a cutaneous form that is most frequent in nature, but often benign, as well as the more aggressive intestinal and inhalational forms, involving a septic state culminating in death. In particular, we will focus upon inhalational anthrax throughout this review.

The 'classical model' of inhalational anthrax pathogenesis was first proposed by Ross after her pioneering efforts during the 1950s [25]. These experiments involved guinea pig phagocytes (mainly macrophages) from the lung, which capture spores and transport them to the thoracic lymph nodes (TLNs), where germination occurs. Within 1 h, spores are taken up by lung phagocytes that then migrate towards the peribronchiolar–perivascular lymphatic channels. Within 4 h, *B. anthracis* spores are detectable in phagocytes located in the TLNs. After 24 h, unrestricted bacillus proliferation is observed in the blood, but Ross did not report recruitment of polymorphonuclear neutrophils or lung injury at any time [25]. Studies on rhesus monkeys [26,27] and chimpanzees [28] infected with spore aerosols confirm that *B. anthracis* is taken up by alveolar macrophages and carried to regional (mediastinum) TLNs within 6–18 h. Interestingly, macroscopic and pathological analyses of inhalational anthrax in humans show that spores are carried by phagocytes accumulating in the capsular sinus of the TLNs [29–32]. It is in the latter setting that spores germinate and bacilli proliferate, producing quite distinctive chains, which then diffuse throughout the circulatory system.

Several recent findings derived from mouse models for anthrax seem to challenge the classical paradigm of alveolar, macrophage-driven pathogenesis and further define certain cellular aspects of the disease. For example, studies with a bioluminescent strain of *B. anthracis* have given a plausibly different picture of the infection kinetics compared with those put forth by previous studies [33]. In this model, bacilli are readily tracked during infection. Either aerosol or intranasal inoculation with spores of this atoxigenic, capsulated strain promotes initial proliferation in the nasopharyngeal area within the nasal-associated lymphoid tissue and mandibular lymph nodes. Vegetative *B. anthracis* are secondarily found in the blood and eventually appear in lungs via the circulatory system. Although resolution of this dynamic technology does not allow intricate analysis of host cell–pathogen interactions, it does provide an important whole-body image of the major steps during infection. Evidently, *B. anthracis* exploits several ports of entry into the body, such as the upper mucosa in nasal-associated lymphoid tissue and lower mucosa throughout the lungs. It is not clear whether the upper entry port is a rodent peculiarity or whether such findings have been missed during analysis of human gross pathology. More detailed studies are needed to determine whether spores found in the TLN participate in disease dissemination. A very recent observation by another group, made possible by using a different luminescent construct of *B. anthracis* controlled through a germination promoter, describes direct germination of spores in the lung alveolar macrophages [34]. Although quite intriguing, this latter finding does not fit any existing model previously described for anthrax pathogenesis.

At the cellular level, numerous studies have attempted to identify cell subsets that transport *B. anthracis* from the alveoli into the regional TLNs. Several experiments reveal the crucial role played by alveolar macrophages during the early events immediately after infection, including the scavenging of spores [35] and sporicidal activity [36]. We have demonstrated recently, with the acapsulated *B. anthracis* spores of Sterne, that conventional lung dendritic cells (DCs) play a major role, in contrast to alveolar macrophages, for capturing and transporting spores to the TLNs [35,37]. Based upon these reports, it is plausible that DCs represent a 'Trojan horse' for spore incubation and subsequent dissemination throughout the body [35]. Such results are to be placed in a more complex context regarding lung interactions with an 'atypical' pathogen, such as *B. anthracis*. Alveolar macrophages, DCs and lung epithelial cells play very coordinated roles that still require further dissection for a more comprehensive understanding of anthrax pathogenesis [38]. For example, it remains unclear if the uptake and transport of *B. anthracis* throughout the host becomes amplified by the bacterium for its own survival. In this regard, edema toxin could accentuate the migration capability of phagocytic cells through upregulation of syndecan-1 and several genes downstream of the cAMP response element binding protein (CREB) [39].

Finally, molecular interactions of the exosporium with various cell subsets have been more closely examined lately, highlighting very specific interactions between professional phagocytes and spores. Studies with a defective *B. anthracis* mutant for *Bacillus* collagen-like protein of *anthracis* (BclA), one of the major exosporium glycoproteins that conformationally mimics the C1q component of complement [40], reveal that BclA specifically targets spores toward professional phagocytes (mainly macrophages) [41]. BclA (21 kD) has a collagen-like, proline-rich sequence and is a member of the TNF family characterized by a trimeric, jelly roll fold. Cell-type selectivity could be important for delaying host death, thus enabling higher production of bacilli (and then spores) for subsequent dissemination into new hosts. The protein core of BclA represents an immunodominant spore antigen [42,43] and, perhaps, BclA is a readily exploitable target for subsequent vaccine and/or therapy endeavors. It is also plausible that BclA acts as an immune decoy with little, if any, vaccine or therapy potential. Interestingly, the carbohydrate moieties of BclA may also be immunogenic [44]. Another recent report describes the complement receptor (CR)3, also known as Mac-1 or CD11b/CD18, as the specific receptor for anthrax spores [45]. However, the authors also claim an alternative uptake mechanism independent of Mac-1, thus there are apparently multiple methods that *B. anthracis* spores employ to enter different cell types.

Clearly, a better understanding of *B. anthracis* pathogenesis and employed virulence factors becomes critical in controlling anthrax from a human vaccine perspective more effectively. The following

section highlights one type of virulence factor, protein toxins, used by *B. anthracis* for survival and subsequent propagation in a host. These same toxins, particularly the PA component, have also been exploited by humans as primary targets for vaccines and therapeutics against anthrax.

B. anthracis toxins: important 'A–B'-based virulence factors

The biochemistry of the *B. anthracis* toxin components has been described extensively in previous reviews [21,23,46], and such detail is certainly not the goal of this current effort. However, we will necessarily focus upon the basic structure and function of PA as it plays a central role during anthrax. Clearly, and understandably so, the PA molecule has become a focal point for many vaccine-based efforts in the past and present. Should this be the case for the future?

Structures & functions of PA

Protective antigen shares structural similarity and function, with many other cell-binding 'B' components produced by *Bacillus* and *Clostridium* spp. [21]. Some toxins produced by these related genera fit a classic binary 'A–B' paradigm, in which the enzymatic 'A' and cell-binding 'B' components are not linked in solution but are synthesized by the bacterium as distinct proteins. The PA precursor (PA83; 83 kD) secreted by *B. anthracis* is proteolytically activated outside of the bacterium into PA63 (63 kD) by trypsin, an unidentified protease(s) in serum, or a ubiquitous cell-surface protease(s) (furin or furin-like) that recognizes a consensus sequence ($_{164}$RKKR$_{167}$) on PA83 [47–49]. Although the 20-kD peptide (PA20) generated after proteolysis of the PA83 precursor slows PA63 clearance from the cell surface via noncovalent interactions (K_D ~190 nM), PA20 does not form membrane channels like PA63 [50]. As per gene expression studies, the PA20 molecule evidently induces apoptosis among human peripheral blood mononuclear cells [51]. Other recent studies show that furin-site mutants of PA83 remain at higher concentrations (vs wild-type PA83) throughout the circulatory system for up to 6 h [47]. Prolonged residence of PA on the cell surface optimizes EF and LF docking opportunities for intoxication. Following proteolysis, either in solution or on the cell surface, PA63 readily assembles into sodium dodecyl sulfate-resistant, hydrophobic homoheptamers. These large structures form pH-dependent (pH < 7), ion-permeable channels in membranes that are readily obstructed by known channel blockers [52,53].

Another unique aspect of the PA63 heptamer is that it provides a cell-surface docking site for two enzymatically distinct proteins, EF and LF, which then respectively form edema and lethal toxin [54]. Both EF and LF possess a unique N-terminal heptapeptide (VYYEIGK) that is integral for competitive docking interactions with PA63 [55]. Each PA63 heptamer can accommodate up to three molecules of EF and/or LF [56]. The VYYEIGK sequence does not appear in the enzymatic components of other bacillus or clostridial binary toxins, further supporting previous data showing that PA does not internalize heterologous A molecules [57]. By contrast, A components from different clostridial binary toxins readily generate biologically active chimeras with heterologous B components [21]. This information, along with a remarkably similar sequence identity, is highly suggestive of a 'successful' virulence factor shared by different *Bacillus* and *Clostridium* spp. From an ecological perspective, these Gram-positive, spore-forming bacilli with anaerobic capabilities reside in similarly diverse niches, such as soil and the gastrointestinal tracts of various animals as well as humans.

X-ray crystallography of PA83 has provided invaluable information on the function of this oligomer-forming molecule (Figure 1) [53]. The PA protein contains domains 1 (N-terminus), 2, 3 and 4 (C-terminus), respectively, involved in docking to an enzyme component(s), channel formation in lipid membranes plus providing an accessory docking site for receptor, oligomerization and binding to specific cell-surface receptor(s) [23]. Domain 1 (residues 1–249) of PA83 contains a cleavage site that subsequently triggers, after proteolysis, release of the N-terminal PA20 and generation of PA63 heptamers [49]. The remaining segment of domain 1 (designated as 1′ and consisting of residues 168–249) faces the channel lumen, unlike the peripherally located domain 4 [53]. Domain 1′ contains two calcium molecules (coordinated via residues D177 or D235 and D179, D181, E188) that evidently preserve a PA63 structure necessary for proper folding, resistance towards further proteolytic degradation, heptamer formation and docking with EF and/or LF [58]. Some monoclonal antibodies toward domain 1 can prevent proteolysis of PA83 into PA63, thus effectively thwarting the intoxication process [59]. The importance of domain 4 (amino acids 596–735) in PA binding to cells was first elucidated by epitope mapping with monoclonal antibodies [60]. Mutagenesis efforts by other groups show that Y681, N682, D683 and P686 represent key residues on PA for binding to receptor [61,62]. Further evidence that an exposed loop on PA (residues 703–722) is important for cell binding was obtained via deletions of nine or 16 amino acids from this region [63]. Other studies reveal that truncations of only five to 12 amino acids from the far C-terminus of PA effectively prevent interactions with cells [64], suggesting an important role in direct binding to the cell surface and/or conformational integrity of PA [65].

Two related, cell-surface receptors that interact with PA83 have been identified: tumor endothelium marker 8 (also known as anthrax toxin receptor [ANTXR]1) [66] and human capillary morphogenesis protein (ANTXR)2 [67], which naturally bind to collagen α3 and collagen IV/laminin, respectively [23]. The crystal structure of PA bound to the von Willebrand domain A and integrin-like domain 1 of ANTXR2 reveals that domain 1 contacts PA domain 4 and a loop (residues 340–380) within domain 2 [68]. ANTXR1 and ANTXR2 contain a metal ion-dependent adhesion site coordinating a divalent cation, which plays a role in PA–receptor interactions [68,69]. Each PA heptamer can bind up to seven receptor molecules [69]. Receptor residues located in the integrin domain 1 and interacting with PA domain 2 contribute to the different affinities of ANTXR1 and ANTXR2 [70]. There are respective K_Ds of 1100 nM and 130 nM for PA binding to magnesium or calcium complexes of ANTXR1; however, there are rather different K_Ds of 170 and 780 pM for PA interactions

with magnesium or calcium complexes involving ANTXR2, respectively [71]. By contrast, another recent study suggests only a threefold difference for PA–ANTXR affinity with K_Ds of 9.5 nM for ANTXR1 and 3.5 nM for ANTXR2 [72]. By using a D683K mutant of PA that interacts specifically with ANTXR2, it has been shown that lethal toxin-induced death in rats is primarily dependent upon ANTXR2 [70]. A low-density lipoprotein receptor-related protein (LRP6) reportedly acts as coreceptor for PA [73], although these results are now rather controversial [74,75].

As described previously, PA83 is readily converted into PA63 and homoheptameric, mushroom-like structures by serine-type proteases in solution or on cell membranes (Figure 1B). Oligomerization of PA63 on cells involves lipid rafts, also known as detergent-resistant microdomains [46]. Once bound to the PA heptamer, EF and LF are then 'threaded' from an acidified endosome through the heptamer-generated pore in an amino-to-carboxy orientation towards the cytosol [76]. Use of polyethylene glycol polymers suggests the pore size to be less than 2 nm [77], which is in agreement with earlier crystal-based studies showing an inner pore diameter of approximately 35 Å [53]. In addition to direct effects upon PA, a low endosomal pH may also facilitate unfolding of EF and LF, which, in turn, promotes translocation into the cytosol [78,79]. Crystal structures of ANTXR2–PA complexes reveal that this receptor acts as a molecular clamp, binding domains 2 and 4 of PA, to restrict toxin pore formation at neutral pH [68]. Data have further shown that the pH threshold for pore formation is lower for ANTXR1 (pH 6.2) than ANTXR2 (pH 5.2) [80,81], leading to an hypothesis that the two receptors may deliver A components into the cytosol at various stages during the endosomal pathway.

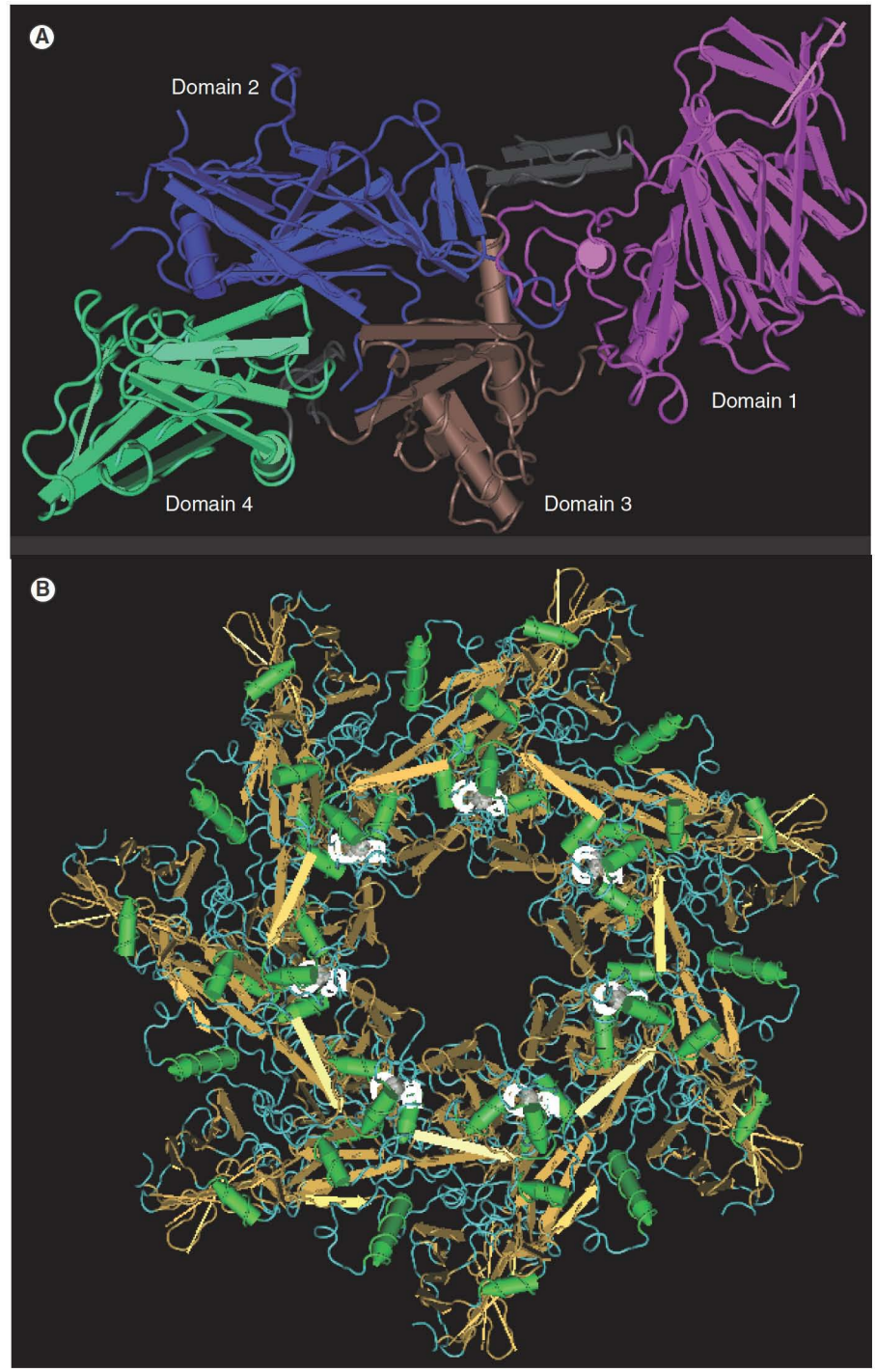

Figure 1. Crystal structures of protective antigen. (A) Monomeric structure of protective antigen (PA83) showing the four domains. Domain 1 docks to an enzyme component (edema or lethal factor), domain 2 forms a channel in lipid membranes and provides an accessory docking site for receptor, domain 3 facilitates oligomerization and domain 4 interacts with cell-surface receptor. **(B)** Heptameric form of PA63 solution – side view.
Structures were derived from data from [53,69] provided by Entrez's 3D database and software for molecular modeling [184].

Domain 2 of PA contains a greek-key β-barrel, consisting of residues 262–368, which unfolds into a β-hairpin, amphipathic loop (residues 302–325). The latter inserts into the membrane, thus promoting an acid-driven conversion of PA from a prepore to pore configuration [82]. This process naturally happens for other *Bacillus* and *Clostridium* binary toxins in an intoxicated cell, specifically within an acidified endosome. Further investigations of domain 2 (encompassing PA residues 259–487) identify D425 and F427 as critical for channel formation and translocation [83]. These same amino acids

are conserved in B components of the clostridial binary toxins [21]. Analyses of PA63 crystals produced at pH 6 and 7.5 show that residues 342–355 become exposed at lower pH [53], thus promoting a more hydrophobic state and oligomerization [84]. It is also within domains 2 and 3 that alanine substitutions of Q277 (buried) and F554 (surface-exposed), respectively, increase thermostability of wild-type PA, which might be useful in improving vaccine stability [85].

Recent data using an F427A mutation of PA indicate that the heptameric pore does not serve simply as a passive conduit, but actively controls EF and LF passage across a membrane [86]. Mutation of F427 effectively inhibits translocation of EF and LF. As F427 is positioned into the channel of a PA63 heptamer, it has been proposed that this residue from each monomer forms a 'phenylalanine clamp' facilitating EF and LF translocation via a 'Brownian ratchet' mechanism [23]. Essentially, the PA63 heptamer acts as a secreted proton–protein transporter akin to other membrane-bound symporter systems commonly used by both prokaryotes and eukaryotes for maintaining homeostatsis.

Role of B. anthracis *toxins during anthrax*

The anthrax toxins represent a model for bacterial-induced suppression that targets both the innate and adaptive immune systems [87]. As with PA, crystal structures for the LF [88] and EF [89] components of the respective lethal and edema toxins have also been quite informative. We will restrict our efforts to the basic effects of these toxin components on host cells and signaling systems. After translocation from an acidified endosome, EF remains bound to the late endosomal membrane while LF is released into the cytosol (Figure 2) [90]. EF is a calcium- and calmodulin-dependent adenylate cyclase that increases intracellular cAMP levels [91]. By contrast, LF is a zinc metalloprotease that cleaves most isoforms of MEKs near the N-terminus [92–94]. Additionally, LF may have other less-defined roles that affect *B. anthracis* pathogenesis. LF inhibits activation of numerous immune cells, including polymorphonuclear cells [95], monocytes [96], macrophages [97,98], DCs [99,100], T cells [101] and B cells [102]. The effects of LF on macrophage survival reveals the different susceptibilities between mouse strains [103] independent of MEK cleavage [104], leading to identification of the inflammasome component NALP1b as a potential target of LF [105]. It has also been shown that other bacterial products (i.e., capsule-derived PGA, lipopolysaccharide, or peptidoglycan) can trigger macrophage sensitivity to LF via TNF [106]. Inflammasome formation involves NALP1 and NOD2 association to activate caspase-1 [107]. LF may induce apoptosis of macrophages through p38α-dependent and -independent pathways [108]. Very recent studies have revealed a role for proteasomes, potassium efflux and caspase-1 activation in LF-induced cell death [107,109]. Maturation of DCs modifies caspase-1-induced death, thus, rendering these cells insensitive to LF-induced apoptosis [110]. Several mitochondrial, as well as inhibitors of apoptosis family, proteins are involved in controlling LF-induced cell death [111,112]. EF also disrupts monocyte [113] and DC [37,100] functions, alone or in cooperation with LF.

The effects of anthrax toxins begin inside *B. anthracis*-containing phagosomes and extend distally throughout the host. The edema and lethal toxins impact disease progression, from early to late stages. Three main phases can be described [38]:

- An invasion phase at the port of entry, whereupon toxins have short-distance effects upon the host
- A phase consisting of bacterial proliferation in the secondary lymphoid organs, with deleterious effects mainly localized to immune cells
- A terminal diffusion phase comprised of elevated toxin levels circulating throughout the bloodstream, with long-distance effects upon numerous organs that ultimately lead to death

During the early stages of anthrax, toxins act primarily as anti-inflammatory products blocking recruitment of proinflammatory cells, thus inhibiting both innate and adaptive immune cell functions critical for maintaining host health [38,87]. Later, when *B. anthracis* toxins are released at high levels into the bloodstream, these proteins disrupt endothelial cell functions and induce shock plus cardiac dysfunctions that cause significant morbidity and mortality [114].

From the physical nature and induced pathophysiology of *B. anthracis* toxins, we now transition into the use of toxin components, in particular PA, as vaccine targets. Although historically used for human vaccines, does PA really represent the best immunogen available for protection against anthrax?

Early development of acellular, PA-based vaccines for humans: the first generation

Gladstone was the first to report, more than 60 years ago, that PA is a crucial target and adequate immunogen against anthrax in various animal models that include mice, guinea pigs, rabbits, sheep and monkeys [115]. Further studies, with more purified forms of PA precipitated on alum, induced protection in guinea pigs, rabbits and monkeys against various virulent strains of *B. anthracis* [116–118]. A preparation of aluminum hydroxide gel-adsorbed PA, now known as anthrax vaccine adsorbed (AVA), has been thoroughly tested for immunogenicity, provides very good protection in various animal models and is readily tolerated in humans [119]. This vaccine effectively protects against cutaneous anthrax among mill workers processing imported goat hair [7]; however, results were not conclusive regarding efficacy towards inhalational anthrax because of the very low, natural incidence of disease. Uniquely, AVA is the only aluminum-based vaccine licensed by the US FDA that is administered subcutaneously [9].

Slight differences have always distinguished the acellular, PA-based vaccines produced on both sides of the Atlantic Ocean [4]. The AVA product made in the USA consists of a cell-free filtrate (formalin treated) from an acapsulated, non-proteolytic derivative of strain V770-NP1-R grown under microaerophilic conditions. The original *B. anthracis* isolate used for AVA production was obtained from a bovine case (1951) of anthrax in Florida, USA. By contrast, the UK vaccine

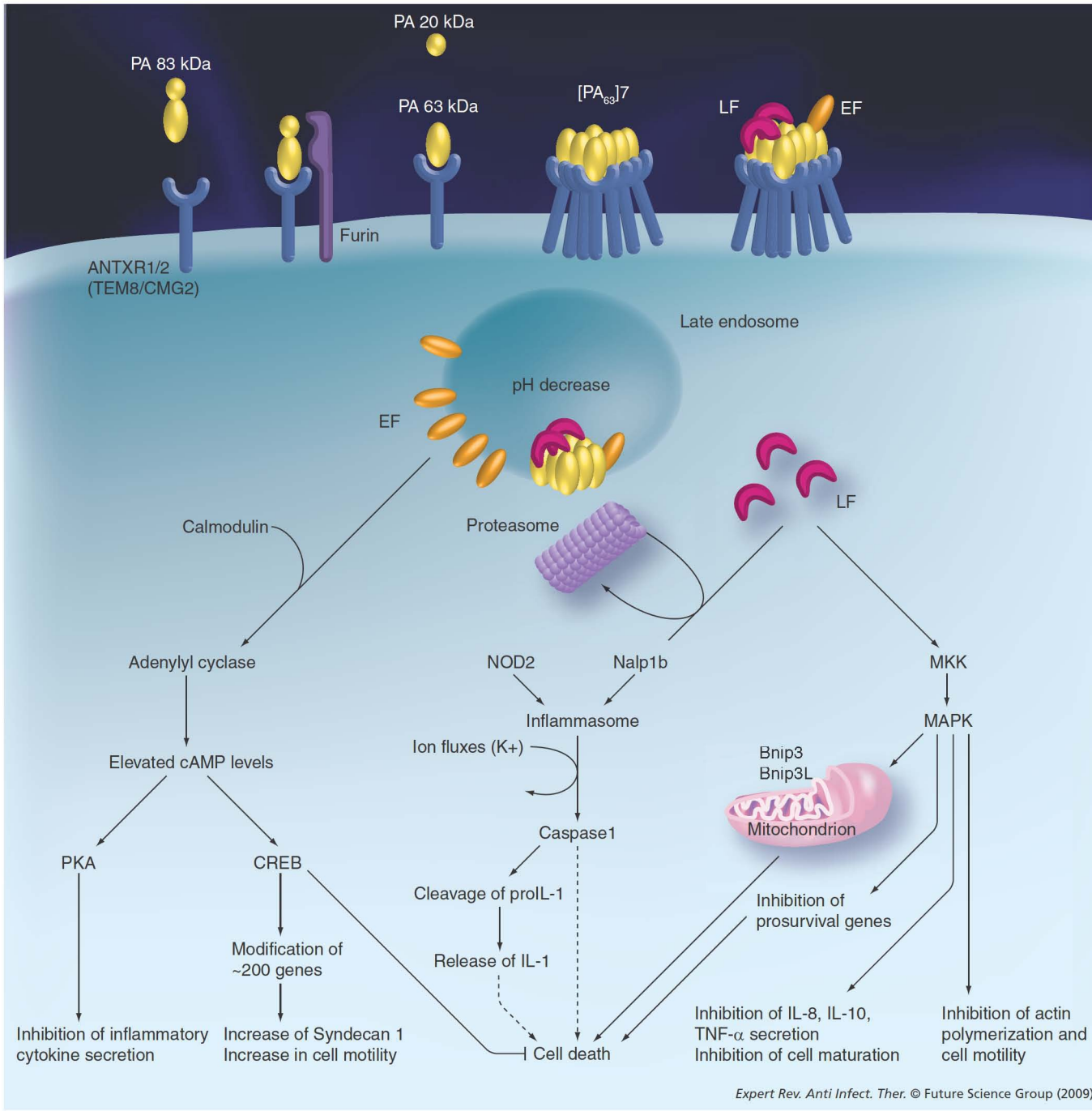

Figure 2. Model for the intracellular effects of lethal and edema toxins. After PA83-ANTXR interactions, PA83 is cleaved by a cell-surface protease (furin or furin-like) and PA20 subsequently released from the complex. Via lipid raft associations, PA63 forms homoheptamers that associate with three molecules of EF and/or LF. LF and EF are translocated with PA into the late endosome. LF is released into the cytosol, while EF remains associated to the late endosomal membrane. EF is a Ca^{2+}–calmodulin-dependent adenylate cyclase that increases cAMP levels and induces cellular dysfunctions through PKA and CREB. Although LF cleaves all MKKs, MKK5 induces major cell dysfunction and death through MAPK pathway deregulation. NALP1b is another critical target for initiating cell death through caspase 1 and IL-1 secretion.
CREB: Cyclic AMP response-element binding protein; EF: Edema factor; LF: Lethal factor; MKK: Mitogen-activated protein kinase kinase; PA: Protective antigen; PKA: Protein kinase A.

against anthrax is an alum-precipitated (anthrax vaccine precipitated [AVP]), cell-free filtrate of Sterne strain (34F2) cultured under static batch conditions with activated charcoal that increases PA production. In addition to PA, both AVA and AVP contain contaminants, such as LF, EF and cell wall proteins; however, much larger quantities of contaminants are present

in AVP [4,120]. As a not so surprising consequence, AVA and AVP vaccinees develop humoral immunity against PA, LF and probably other *B. anthracis* antigens [121].

Second-generation PA-based vaccines: a recombinant product

In an attempt to generate a less-heterogenous vaccine against anthrax, it has been over a decade since recombinant PA (rPA) was first shown to be immunogenic and protective against a spore challenge in a guinea pig model [122]. Subsequent studies by various groups have demonstrated rPA efficacy in association with adjuvant for systemic or mucosal routes of immunization in several animal models that include mice, rabbits and monkeys [12,123–125].

A logical extension of these earlier animal experiments with rPA is the current clinical development of a second-generation vaccine for anthrax, consisting of rPA as the sole immunogen. The vaccine, designated as PA102 and produced by a *B. anthracis* strain lacking both virulence plasmids, is immunogenic and well tolerated as evidenced by a recent Phase I clinical trial [126]; however, some concerns have been expressed regarding study design [127,128]. Although safe and homogeneous, overall immunogenicity of PA102 was deemed no better than that of AVA. Additionally, there have been some unfortunate manufacturing issues linked to stability of the rPA vaccine [10].

How do PA-based vaccines work?

Neutralizing antibodies are the best effectors of the immune system to protect against intoxication [129]; therefore, the classical readout of PA-based vaccines is induction of a specific humoral response. The protective role of neutralizing, PA-specific antibodies elicited by vaccine was demonstrated by early experiments employing hyperimmune animal serum that protects mice against *B. anthracis* spores [115,130]. Many subsequent, concordant studies have shown that mouse, monkey and human monoclonal antibodies against PA can protect against infection that includes the effects of edema and lethal toxins in different animal models [131]. Numerous B-cell, but fewer T-cell, epitopes on PA have been identified by mouse and human immune systems [59,131,132]. The B-cell epitopes are not equally distributed and form clusters within domains 1, 2 and 4. T-cell epitopes are evident throughout the entire PA molecule [131]. Little is known about the role of PA-specific $CD4^+$ T-cells required for antibody-mediated immunity, although this specific subset has been described among humans vaccinated with AVA [133].

According to the localization of B-cell epitopes, PA-binding antibodies could act through interference of:

- LF and EF docking to the PA heptamer
- Channel formation in lipid membrane
- PA interactions with receptor

Most of the B-cell epitopes, especially those that interact with neutralizing antibodies, are found within domain 4. As a further example, immunogenicity of the different domains within PA has been tested, and domain 4 is clearly indispensable for inducing a protective immune response in A/J mice [134]. More recent data comparing the immunogenicity of PA have shown that genetic background (in mice) determines relative efficacy of the immune response, thus also suggesting variable immune responses in human populations [132]. Two limited studies among humans show that AVA immunization elicits antibodies against domain 1 epitopes on PA [135,136]. However, PA20-specific antibodies should have no effect upon PA63 and formation of homoheptamers necessary for binding and subsequent transport of EF and LF into cells.

Beyond the direct neutralization capacity of PA-induced antibodies and subsequent clearance of PA from the body, specific antibodies can also inhibit spore germination, as well as increase the phagocytic–sporicidal activities of macrophages [137,138]. Several vaccine studies report a good correlation between neutralizing antibody titers against PA and protection towards anthrax in mice [139], guinea pigs [140] and rabbits [141–143]. Based upon animal studies, it is not surprising that correlates of immunity derived from serum include ELISA titers against PA plus toxin-neutralization titers in cell-based cultures that have both been adopted for humans vaccinated with AVA [144].

Although PA-based vaccine strategies have been employed by many investigators over time, there are very few data regarding the mechanisms of how PA-binding antibodies protect against an infection *in vivo*. Anthrax, like any other microbe-initiated disease process, is indeed complex and consists of an intricate relationship between intoxication, infection and host response to the offending pathogen and its byproducts. As the *B. anthracis* toxins may act during every stage of anthrax, from spore germination within phagocytes to the terminal stage involving large concentrations of these toxins throughout the circulatory system, it is easily understood that PA-targeting antibodies protect primary immune-cell effectors (macrophages, DCs, B and T cells) from the immunosuppressive effects of these toxins [38]. As a result, vegetative forms of *B. anthracis* are eliminated more readily by animals vaccinated with PA, as shown in a study conducted in the 1960s comparing the bacterial loads in naive versus immunized animals [145]. More efficient phagocytosis of bacilli diminishes toxin release into the bloodstream, ultimately leading to better survival rates. Accordingly, a recent study shows that PA-immunized animals effectively prevent bacterial proliferation at a localized infection site in a subcutaneous model for anthrax [146]. It is clear that an anti-PA immune response efficiently blocks the growth of *B. anthracis* at the port of entry.

PA vaccine immunogenicity & sustainability of the immune response

From the very beginning, human vaccine protocols for anthrax have been rather cumbersome, requiring (for AVA) six subcutaneous injections (0.5 ml each) over an extended period (0, 0.5, 1, 6, 12 and 18 months). The schedule for AVP is slightly different, and consists of four intramuscular injections (0.5 ml each) administered at 0, 0.75, 1.5 and 8 months [147]. Additionally, there are annual boosts for both AVA and AVP, necessary for

maintaining protective immunity. These extensive regimens have been defined according to early animal studies [7]. By quite sharp contrast, the human vaccination procedure may be compared with the single subcutaneous injection (and annual boost) of the veterinary-based Sterne vaccine consisting of spores. Two recent, independent studies demonstrate that extending time intervals between the initial doses of AVP or AVA may increase PA-specific titers [9,148]. Additionally, fewer adverse reactions (i.e., injection site tenderness, erythema, headache and induration) were noted for AVA when time between the first two injections was increased from 2–4 weeks and when administered intramuscularly (vs standard subcutaneous injection). It should also be noted that adverse reaction rates to subcutaneously administered AVA, in comparison to other toxin-based vaccines in use today (i.e., diphtheria, pertussis and tetanus) are equivalent [149]. Marano *et al.* recently published an extensive clinical trial showing that AVA, when administered subcutaneously or intramuscularly (three or four injections), is equivalent to the licensed regimen regarding immunogenicity and safety within 7 months after vaccination [150]. Besides modifications in both the injection sites and intervals with AVA or AVP, is there something from the Sterne vaccine experience that could, and perhaps should, be applied to human vaccinations against anthrax? By noting the 70-year success of Sterne's spore-based vaccine around the world, we propose there is something very important to be gleaned from the veterinary vaccine for improving existing human vaccines against anthrax. For instance, might select antigens, perhaps in addition to PA, afford longer lasting protection in humans?

Recent human studies demonstrate that humoral immune responses against PA decrease rapidly within months after the last boost for both AVA and AVP [150,151]. Early studies show that AVP-vaccinated rabbits are no longer protected 23 weeks post-vaccination [118]. This result was further confirmed in a common model employing rabbits given two doses of rPA with aluminum-based adjuvant. Protection levels against a lethal

Table 2. Strategies for experimental anthrax vaccines.

Type	Antigens	Adjuvant	Route (doses)	Model of infection	Ref.
PA mutant domain	Dominant negative rPA	Aluminum hydroxide adsorbed	ip. (3)	BALB/c mice challenged with lethal toxin	[156]
	rPA domain 4	Alhydrogel	im. (2)	A/J mice infected by Sterne spores (ip.)	[134]
Antigen combination	rPA + PGA	Ribi	sc. (2)	Outbred mice infected by Ames spores (sc.)	[14]
	rPA + PGA	Alum	ip. (3)	BALB/c mice infected by Ames spores (ip.)	[162]
	PA + inactivated spores	Alum	sc. (2)	Outbred mice and guinea pigs infected by strain 9602 spores (sc.)	[13]
	rPA (prime) + BclA (boost)	Titermax Gold (water in oil)	ip. (1 prime and 1 boost)	A/J mice infected by Sterne spores (sc.)	[160]
Adjuvant combination	PA	Aluminum hydroxide adsorbed + CpG	sc. (2)	Macaques immunized and serum transferred to A/J mice infected by Sterne spores (ip.)	[168]
	PA	Aluminum hydroxide adsorbed + CpG	sc. (2)	Guinea pigs infected by Vollum 1B spores (id.)	[167]
	PA	QS-21 MPL	im. (1) im. (1)	Macaques infected by Ames spores (aerosol)	[11]
	rPA	Cholera toxin	in. (3)	None	[170]
	rPA	CpG + EF	sc. (2)	None	[173]
Mucosal	rPA	Poly-L-lactide	in. (2)	A/J mice infected by Sterne spores (ip.)	[125]
	rPA	Aluminum hydroxide adsorbed + CpG	in. (2)	A/J mice infected by Sterne spores (aerosol)	[168]
	rPA	Water-in-oil nano emulsion	in. (2)	Guinea pigs infected by Ames spores (in.)	[177]
	rPA	Dry powder	in. (2)	Rabbits infected by Ames spores (aerosol)	[178]
	Lactobacillus-expressed PA	None	Oral	Under investigation	[183]
Intradermal	rPA	CpG	id. (2)	Rabbits infected by Ames spores (aerosol)	[179]

EF: Edema factor; id.: Intradermal; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; MPL: Monophosphoryl lipid A; PA: Plasminogen activator; PGA: Poly-γ-D-glutamic acid capsule; rPA: Recombinant protective antigen; sc.: Subcutaneous.

aerosol challenge with *B. anthracis* spores dropped from 74% at 6 months to 37% at 12 months after the primary injection of rPA vaccine [152].

One explanation for the short durations of an anti-PA antibody response could be that PA has nanomolar affinity for its ubiquitous cell receptors [71]. It should be noted that this affinity is similar to that for many PA-targeting antibodies described to date [153,154]. As a correlation linked to relative binding affinities, domain 1 of ANTXR2 represents a better receptor-based decoy for inhibiting anthrax intoxication versus the ANTXR1 equivalent [71]. Recent data have proven that PA is very rapidly cleaved in the serum of mice or rats and thus eliminated from the general bloodstream within 6 h [47]. However, circulating PA levels can remain 50-fold higher at 16 h with a PA molecule defective in binding to receptor. So, it can be easily imagined that native PA is adsorbed rapidly by its ubiquitous receptors throughout the body on various cell surfaces. Such events may make long-term immunity targeting PA more difficult to maintain with existing vaccine technologies.

With that stated above, one can easily understand the necessity for a six-shot regimen of PA-based vaccines. It is possible that only a fraction of injected PA becomes detected effectively by the immune system. Perhaps an improved duration of the immune response can be achieved with a defective form of PA (i.e., unable to bind receptor)? A recombinantly attenuated PA, containing modifications in domain 4, could be an option for decreasing PA–ANTXR interactions, although data also show that domain 4 is necessary to induce protective immunity [134]. Clearly, changes within domain 4 would require very careful molecular manipulations that include minimal perturbation of natural epitopes that afford protection towards not only the toxin, but also *B. anthracis* infection.

Third-generation vaccines: which way to go?

Many different strategies have been published in the last decade to improve existing anthrax vaccines for human use (Table 2). We will now focus upon, in our opinion, the most efficient and promising strategies to date. As shown by numerous concordant vaccine studies, PA is central to the control of anthrax, and this molecule evidently constitutes the major target for future human vaccines. We will consider here the modification of PA to improve its immunogenicity, the addition of other *B. anthracis* antigens, the role of novel adjuvants and routes of immunization. The ultimate goal is to develop a more efficacious vaccine for humans against all forms of anthrax that can be administered with minimal follow up (i.e., few, if any, booster injections) and few adverse reactions.

Recombinantly-modified PA: whole molecule vs separate domains?

Clearly, at least to us, there is a need to improve the immunogenicity of PA when used as a lone vaccine component. One strategy consists of bioengineering PA either to increase its availability to the immune system via targeted mutations or producing separate immunogenic domains. Initial studies have yielded a dominant negative form of PA containing mutations in domain 2 (K397D and D425K) that block EF and LF transport into cells [155]. This version of PA has proven itself as a good therapeutic and immunogen in a mouse intoxication model [156]. Versus wild-type PA, the immunogenicity of dominant negative mutants is evidently improved by unknown mechanisms [156]. These mutations could perhaps induce better accessibility of PA for processing and/or enhance presentation to $CD4^+$ T-cells. Such studies represent new strategies to improve PA immunogenicity that would be interesting to evaluate in infection models.

Another strategy involves engineering of separate PA domains of interest, such as domain 4, which is critical for PA neutralization as demonstrated by antibodies or receptor-based decoys [134]. Of course, a concern of this latter strategy would be retention of proper, native conformation and inherent neutralizing epitopes.

PA combined with other antigens: spore vs capsule additions?

Early vaccine studies in animals that compare efficacy of an attenuated spore vaccine, versus PA alone, demonstrate that immune responses against other *B. anthracis* antigens could afford better protection [130,157]. Two main antigen categories are of concern: those forming the spore during early stages of infection and those associated with vegetative bacilli represented mainly by the extracellular capsule.

Formaldehyde-inactivated spore (FIS) antigen in conjunction with PA can induce impressive protection in cutaneous/inhalation models of anthrax infection among mice and guinea pigs [13,158]. Individually, either FIS or PA alone affords rather poor protection against highly virulent strains of *B. anthracis* in these models; however, a combination vaccine consisting of both antigens elicits complete protection. Indirectly, these data suggest complementarities of *B. anthracis* antigens as evidenced by robust, protective immune responses. The same group also demonstrated that protection induced by FIS is related to cellular immune responses linked to IFN-γ-producing $CD4^+$ T cells [159].

In the same way, recent efforts by another group have used non-glycosylated, recombinant BclA (expressed by *Escherichia coli*) plus PA in an A/J mouse model. Animals were initially vaccinated with PA, followed 2 weeks later by a dose of BclA, before a subcutaneous challenge composed of Sterne spores [160]. Either PA or BclA alone affords little protection in this model, yet when combined in the vaccination protocol there is complete protection. This same group also confirmed that the sera of rabbits previously immunized with BclA, when passively transferred to naive mice, only increases the time to death in A/J mice against a lethal spore challenge. However, the anti-BclA antibodies enhance spore capture and decrease intra-macrophage germination *in vitro* [160]. In addition to the BclA protein, attached polysaccharides may also represent an immunogenic component of interest [44]. Overall, FIS or selected spore antigens, such as BclA, could also serve as useful targets that provoke more efficacious humoral and cellular immune responses against *B. anthracis*, ultimately affording excellent protection in several complementary animal models and humans.

Another attractive antigen produced by *B. anthracis* is the PGA-containing capsule encompassing the bacillus, but a major hurdle for its incorporation into a vaccine is poor immunogenicity. However, the latter can be overcome by PGA conjugation to a carrier, but unfortunately the first report of this strategy did not evaluate protection in an infection model [161]. A more-detailed report of PA immunogenicity, alone or in combination with conjugated or unconjugated PGA, shows that the conjugation of PA with PGA is necessary for protection against a fully virulent strain of *B. anthracis* [14]. Conjugated PGA alone also provides partial protection in mice challenged with vegetative bacilli of the Ames strain [162]. From a manufacturing perspective, further development of a PGA-based vaccine may be limited by the amount of PGA that can be produced under good-manufacturing practice conditions.

PA combined with novel adjuvants

Current anthrax vaccines for humans employ aluminum-based adjuvants. The general effects of adjuvants, and how to optimize them, is highly debated and has been reviewed elsewhere [163,164]. Various groups have shown that adjuvants, such as threonyl-muramyl dipeptide, monophosphoryl lipid A (MPL) [165], QS-21 [11] and CpG oligodeoxynucleotides [166] improve humoral immunity towards PA in different animal models. A combination of PA, MPL and the cell wall cytoskeleton of either *Mycobacterium phlei* or BCG strain of *Mycobacterium bovis* induces strong protection in guinea pigs challenged with Ames strain spores [165]. As these adjuvants elicit strong Th1 immunity, such data further confirm the supportive role of cellular immunity for protection against anthrax [159]. CpG oligodeoxynucleotides have also been tested in association with AVA, improving the protective immunity against spore challenge in both nonhuman primates and guinea pigs [167]. A study comparing the effects of CpG with AVA reveals better protection against inhalational anthrax when administered by a systemic, versus mucosal, route [168]. However, CpG can also be toxic in mice and such results seemingly limit their potential use in humans [169]. Finally, bacterial proteins, such as cholera [170], pertussis [171] and edema toxins [172,173], share common effects on eukaryotic cells that include increasing intracellular levels of cAMP. These bacterial proteins can act as effective systemic and mucosal adjuvants that facilitate anti-PA immune responses in mice. However, this latter type of adjuvant may not readily transition into humans due to potential toxicity. Overall, success in animal models with these diverse adjuvants must eventually be translated into human efficacy following lengthy (and expensive) clinical trials.

Evaluation of mucosal immunization against anthrax

Although stimulation of mucosal immunity seems logical for preventing anthrax, the development of novel mucosal adjuvants for human use has been under intense scrutiny for many years [174]. These new adjuvants enable select targeting that can restrict an immune response to the mucosa. Compartmentalization within the mucosal immune system places constraints upon the vaccination route for inducing effective immunity at the desired site. With respect to the lungs as a target, intranasal immunization is seemingly the best route for stimulation [174]. The anatomical site where *B. anthracis* dissemination is blocked in immunized animals has not yet been determined clearly. According to pathogenesis data, effective prevention of anthrax must occur at an early stage before *B. anthracis* spreads from the lungs and throughout the body [145]. Recent data with cutaneous anthrax reveal that proliferation is blocked at very localized regions in immunized mice [146]. Therefore, it may be hypothesized that after effective immunization there is a strong, sterilizing response within the lung immune system. However, identification of various effectors contributing to lung mucosal immunity in humans during a *B. anthracis* infection is incomplete. We have recently discussed in detail the basis of lung immunity during anthrax [175]. Lung immune responses are not only comprised of phagocytes scattered throughout the lung tissue, but also the draining lymph nodes. The mucosal immune system is a growing subfield of immunology and the role of local immunity is well established. Local antispore immune responses may be beneficial by killing the pathogen before it produces toxin, and killing emerging bacilli would have the same effect. Perhaps the role of recently discovered effectors during bacterial infection of the lungs, such as Th17 cells and IL-22, warrant further investigation [176]. Future anthrax vaccines should, perhaps, induce a specific subset of T-helper cells for an optimal, protective response.

Intranasal immunization has indeed been tested by various groups with different formulations. The rPA, when microencapsulated in nanoparticles of poly-L-lactide or absorbed into a water-in-oil nanoemulsion, induces protection against Sterne or Ames strains of *B. anthracis* in mice and guinea pigs [125,177]. A dry-powder vaccine of rPA has also been tested successfully in rabbits for protection against Ames [178,179].

Another interesting strategy against anthrax consists of oral immunization using a live vector. An advantage of this type of mucosal immunization is protection induced in both the lungs and gut, in theory protecting against two forms of anthrax. Several vectors for PA presentation to the gut mucosa have been tested and include live Sterne strain of *B. anthracis* [180], *Bacillus subtilis* [181] and *Salmonella* [182]. All three vectors are capable of inducing a PA-specific immune response. More promising, perhaps, is the use of probiotics generally regarded as safe, such as *Lactobacillus* spp. expressing PA fused to a peptide that targets DCs. This latter approach may constitute a more 'naturally' effective way to afford protection via the gut and lung immune systems [183]. Although many of these methods are seemingly viable in animals, important clinical trials determining safety and efficacy in humans are still pending.

Expert commentary & five-year view

In recent years, many groups have addressed the multitude of issues linked to improving anthrax vaccines for humans. There have certainly been many advances since the first anthrax vaccine was developed for veterinary purposes over a century ago by Pasteur's group. However, in our opinion, we are still far from an optimized anthrax vaccine for humans. For example, multiple injections over many months along with an annual boost

suggest that human vaccines available for anthrax are far from optimal. This is in contrast to the veterinary, spore-based vaccine that requires only one inoculation followed by an annual boost. Recent studies show that an altered vaccination schedule (i.e., fewer injections) with currently licensed vaccines for human use can be effective with fewer side effects. It is clear, at least to us, that in addition to PA other *B. anthracis* antigens should be seriously considered for generating more efficacious vaccines against anthrax. A more-thorough understanding of immunologically pertinent *B. anthracis* antigens, better adjuvants and how an infected host fights anthrax at a cellular level are all critical elements that must be carefully considered by researchers. Clearly, initial conceptualization and then further development of future anthrax vaccines for humans will benefit from recent data linked to anthrax pathophysiology that includes the immune system of the lung mucosa. Parallel development of novel adjuvants and vectors for targeting antigen(s) to local effector cells may also provide exciting tools in the upcoming years. As PA will undoubtedly remain a primary target for the next generation of anthrax vaccine(s), delivery routes may change and include novel adjuvants as well as other crucial antigens of *B. anthracis*. These diverse steps will ultimately contribute to a much better anthrax vaccine and, when finally accomplished, greatly diminish the specter of *B. anthracis* being used as a bioterrorism/biowarfare agent. Simply put, there needs to be a better understanding of anthrax at many levels, which includes both science and politics.

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Key issues

- Current anthrax vaccines for humans require multiple boosts and novel vaccine strategies are necessary.
- Recent studies show efficacy with currently licensed human vaccines when administered with fewer injections and alternative routes.
- Besides protective antigen, other *Bacillus anthracis* antigens should be considered for human anthrax vaccines as a mixed, defined inoculum.
- New-generation vaccines for anthrax should elicit both humoral and T-cell-mediated immune responses for optimal protection.

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